

# Hominids adapted to metabolize ethanol long before human-directed fermentation

Matthew A. Carrigan<sup>a,b,1</sup>, Oleg Uryasev<sup>b</sup>, Carole B. Frye<sup>b</sup>, Blair L. Eckman<sup>b</sup>, Candace R. Myers<sup>c</sup>, Thomas D. Hurley<sup>c</sup>, and Steven A. Benner<sup>b</sup>

<sup>a</sup>Department of Natural Sciences, Santa Fe College, Gainesville, FL 32606; <sup>b</sup>Foundation for Applied Molecular Evolution, Gainesville, FL 32604; and <sup>c</sup>Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202

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**Paleogenetics is an emerging field that resurrects ancestral proteins from now-extinct organisms to test, in the laboratory, models of protein function based on natural history and Darwinian evolution. Here, we resurrect digestive alcohol dehydrogenases (ADH4) from our primate ancestors to explore the history of primate-ethanol interactions. The evolving catalytic properties of these resurrected enzymes show that our ape ancestors gained a digestive dehydrogenase enzyme capable of metabolizing ethanol near the time that they began using the forest floor, about 10 million y ago. The ADH4 enzyme in our more ancient and arboreal ancestors did not efficiently oxidize ethanol. This change suggests that exposure to dietary sources of ethanol increased in hominids during the early stages of our adaptation to a terrestrial lifestyle. Because fruit collected from the forest floor is expected to contain higher concentrations of fermenting yeast and ethanol than similar fruits hanging on trees, this transition may also be the first time our ancestors were exposed to (and adapted to) substantial amounts of dietary ethanol.**

experimental paleogenetics | alcohol dehydrogenase | ethanol | primates | evolution

One trend in modern medicine attributes diseases in humans to an incomplete adaptation of the human genome to new challenges presented by our changing cultural and demographic environment (1). This attribution is especially convincing for some “lifestyle” diseases. For example, the recent increase in sugar consumption (including sucrose and fructose) is associated with the emergence of obesity, diabetes, and hypertension (2). Under an evolutionary paradigm, an organism fully adapted to a sugar-rich diet would not be expected to become diseased by consuming sugars, suggesting that humankind has not had enough time to adapt to a modern diet rich in such sugars.

It is unclear whether the human genome has had more time to adapt to dietary ethanol (“alcohol” in the vernacular), which also produces a disease spectrum (“alcoholism”) common today in many societies (3). In one historical model, ethanol was not a significant part of the hominin “Paleolithic diet” (4) and was also absent from the diets of earlier ancestors. Rather, the model holds that ethanol entered our diets in significant amounts only after humans began to store surplus food (possibly because of the advent of agriculture) and subsequently developed the ability to intentionally direct the fermentation of food (~9,000 y ago (5), perhaps as a means of preservation (6). In this model, alcoholism as a disease reflects insufficient time since humans first encountered ethanol for their genome to have adapted completely to ethanol. As such, the allelic variants of enzymes in the ethanol metabolic pathway that disfavor ethanol consumption (e.g., *ADH1B\*47His* and *ALDH2\*487Lys*, both of which lead to an accumulation of acetaldehyde—a toxic intermediate that causes headache, nausea, and general discomfort) represent an early stage of adaptation, possibly in association with pathogenic infections (7–10).

In an alternative model, primates may have ingested ethanol via frugivory as early as 80 million y ago (Ma), a time corresponding to the origin and diversification of primates (11) and

when angiosperm plants first produced fleshy fruits that can become infected by yeast capable of the accumulating ethanol via fermentation (12). In one version of this model, small amounts of ethanol present in slightly fermenting fruit attached to trees attracted arboreal primates foraging in the trees. In this version, our contemporary attraction to ethanol is an “evolutionary hang-over” that ceased to be beneficial once that attraction became redirected to beverages with high concentrations of ethanol (13), made possible only after humans developed the tools allowing them to intentionally direct fermentation (and enhanced with the advent of technology to distill ethanol to higher concentrations). Another version of the “ethanol early” model for ethanol exposure recognizes that ethanol itself, as well as the food naturally containing it, can be a significant source of nutrition. This model posits that any organism with metabolic adaptations that permit the exploitation of ethanolic food would have access to a specialized niche or important fallback foods unavailable to organisms without this metabolic capacity.

Paleogenetics is an emerging field designed to address such natural historical hypotheses and, in particular, to distinguish between competing historical models (14). Here, to gain a genetic perspective on the natural history of the interaction between our human ancestors and ethanol, we examined the evolution of Class IV alcohol dehydrogenases (ADH4) (see *SI Text* for a discussion of the various synonyms used within the ADH family). These digestive enzymes are abundant in the stomach, esophagus, and tongue of primates and are active against a wide range of alcohols. Thus, ADH4 is the first alcohol-metabolizing enzyme to

## Significance

Many modern human diseases are attributed to incompatibility between our current environment and the environment for which our genome is adapted. It is unclear whether this model applies to alcoholism. We investigated this possibility by studying alcohol dehydrogenase class IV (ADH4), the first enzyme exposed to ethanol in the digestive tract that is capable of metabolizing ethanol. We resurrected ancestral ADH4 enzymes from various points in the ~70 million y of primate evolution and identified a single mutation occurring ~10 million y ago that endowed our ancestors with a markedly enhanced ability to metabolize ethanol. This change occurred approximately when our ancestors adopted a terrestrial lifestyle and may have been advantageous to primates living where highly fermented fruit is more likely.

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See Commentary on page 308.

<sup>1</sup>To whom correspondence should be addressed. Email: [matthew.carrigan@sfcoll.edu](mailto:matthew.carrigan@sfcoll.edu).

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encounter ethanol that is imbibed (15), and several studies indicate that ADH4 contributes significantly to the first-pass metabolism of ethanol in humans (16).

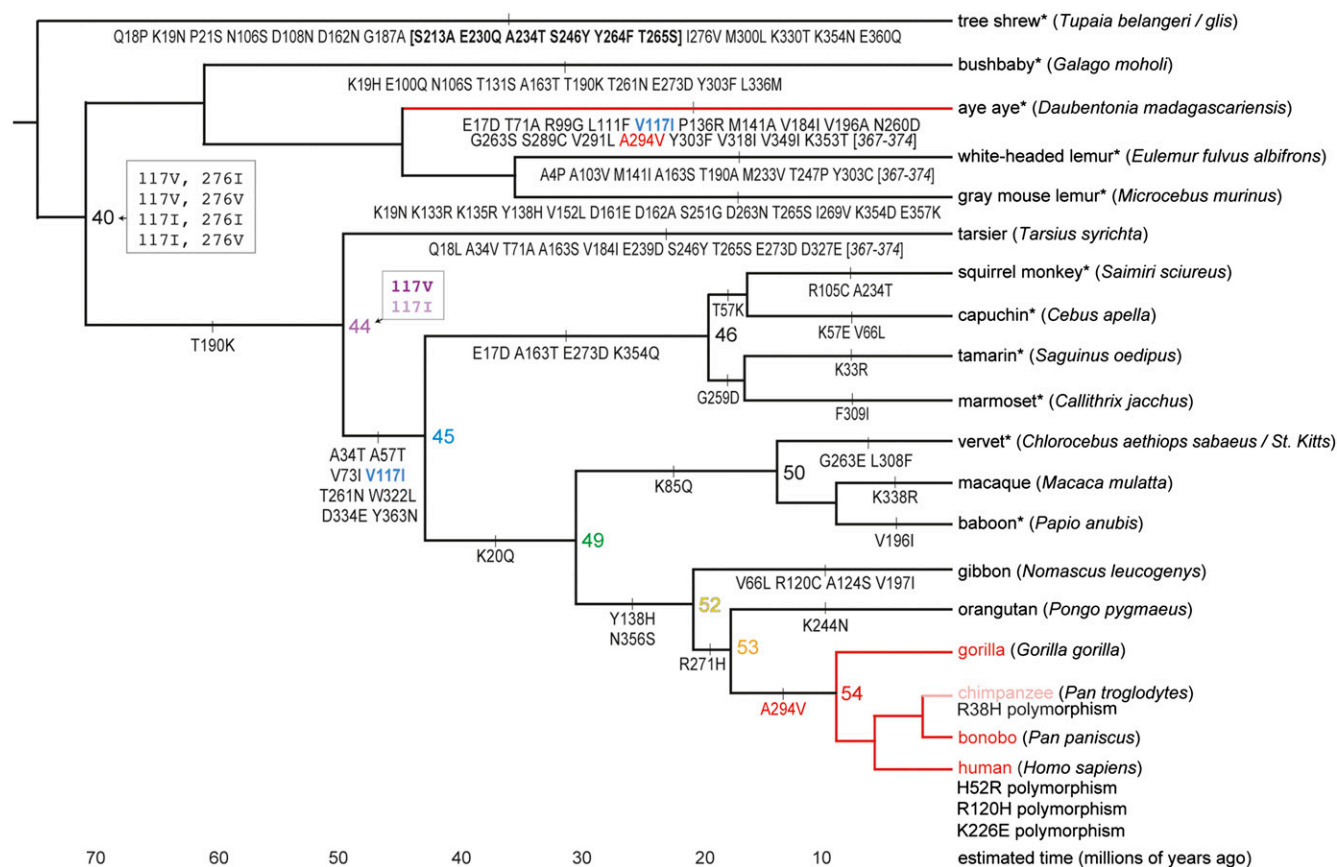
ADH4 is also active against retinol (in vitro), and ADH4's high catalytic efficiency for retinol (as defined by its  $k_{cat}/K_M$  ratio) suggests it may play a role in retinoic acid biosynthesis (17). Mice with inactivated ADH4 genes, however, display few complications associated with retinoid metabolism except under extreme conditions of dietary retinol excess or dietary retinoid deficiency (18, 19). Further, dietary retinoids occur in the form of retinyl esters (from animal foods) or carotenoids (from plant foods); these forms of provitamin A are not substrates for ADH4 present in the upper gastrointestinal track and are converted into retinol only after entering the small intestines. Geraniol, however, is a monoterpene that is structurally similar to retinol and is commonly found in plants as an antifungal, making it a physiologically relevant substrate for ADH4 in herbivorous primates.

We therefore tested alternative models for the history of primate exposure to ethanol by comparing the enzymatic efficiencies of modern and ancestral ADH4 enzymes toward geraniol and ethanol. These comparisons identified a dramatic evolutionary transition from an ethanol-inactive ADH4 to an ethanol-active ADH4 in our hominin ancestors ~10 million y ago.

This study focuses on the evolution of one component of ethanol metabolism, ADH4. Ethanol metabolism is complex and involves other ethanol-metabolizing enzymes [e.g., ADH1, ADH2, and the microsomal ethanol oxidizing system (MEOS)], enzymes involved in the downstream metabolism of by-products from ethanol metabolism (e.g., ALDH2, which oxidizes acetaldehyde created from ethanol), and enzymes indirectly affected by the by-products of ethanol metabolism (e.g., ALDH1). A more nuanced understanding of primate adaptation to ethanol will develop as future work examines these related enzymes.

## Results

A maximum-likelihood analysis (20) was used to infer the sequences of nine ancestral ADH4 proteins spanning the ~70-million-y history of the primate Order (Fig. 1). These ancestral proteins were synthesized and purified, and their kinetic properties were examined. The evolution of the behaviors of these ancestral enzymes is striking. Nearly all digestive ADH4 enzymes from our primate ancestors were largely inactive against ethanol (Table 1). They did, however, oxidize other alcohols (Table S1), including terpenoid alcohols (such as geraniol) (Table 1) that are abundant in the leaves of plants (21). These results suggest that the last common ancestor of humans and orangutans (node 53 in Fig. 1) was unable to efficiently metabolize dietary ethanol



**Fig. 1.** The reference phylogeny of the alcohol dehydrogenase 4 (ADH Class IV, ADH4) sequences used in this study. Amino acid changes in the evolution of ADH4 proteins are shown along tree branches. Ancestral ADH4 proteins examined in this study are indicated by numbered nodes within the tree. Ambiguities at nodes are indicated within gray boxes; for example, the sequence at node 44 contained one ambiguity at site 117 (the posterior probabilities for this site were 86% for valine and 14% for isoleucine). All ambiguous versions of the ancestral proteins were resurrected and found to have similar kinetic properties. Blue and red text below branches indicate the V117I and A294V homoplasies shared by the aye aye and Homininae lineages. Sequences indicated by \* were, to our knowledge, determined for the first time in the research described here. Sections of gene sequences that are missing in the genomic data are indicated by the corresponding amino acid numbers in italics (except in the case of *T. belangeri*, where the missing region, indicated in bold, was substituted with the corresponding region of the closely related *T. glis*). Some of the polymorphisms found in humans and chimpanzees are indicated on the tree below each species' name. Branches of the tree in red indicate enzymes active against ethanol. The branch leading to chimpanzee is pink to indicate that the R38H polymorphism is frequent among *Pan troglodytes* (based on the limited sequences in current genomic databases), and the impact of this polymorphism is not known.





fragmented forests and grassland ecosystems (29–32), and coincided with a wave of extinctions (33, 34). Among other global extinctions, hominoid fossils once abundant in the early Miocene forests of East Africa become rare in the middle and late Miocene grassland ecosystems (31, 35, 36).

The major ecosystem changes and mass extinctions seen after the middle Miocene climatic transition suggest changing selective pressures that are associated with adaptations to novel ecological niches (37), including increased terrestriality in the HCG ancestor. Our hominoid ancestors living in the early Miocene and Oligocene (before the MMCT), including the last common ancestor shared with cercopithecoids, were almost certainly arboreal frugivores (38–40). Early Miocene (17–22 Ma) hominoids, such as *Proconsul* (41), and several hominoids of the middle Miocene, including *Nacholapithecus*, were also arboreal frugivores (42–45). Although modern humans, chimpanzees, and gorillas have maintained a frugivorous diet, they have since evolved terrestrial locomotor adaptations and (at least occasionally) consume food collected from the ground (46, 47). Indicators of knuckle-walking in the fossils of *Kenyapithecus* provide evidence of semiterrestriality in hominoids as early as 14–16 Ma (48–50). Fossil evidence suggests that terrestrial adaptations were present in early hominids [e.g., *Orrorin*, 6 Ma (51, 52), and *Sahelanthropus*, 7 Ma (53, 54)], close to the estimated divergence of the human–chimp/gorilla ancestor. Thus, the appearance of an ethanol-active ADH4 in the HCG ancestor occurred at approximately the same time these ancestors were adapting to terrestrial life (presumably) in response to ecological changes brought about by the MMCT.

Saps, nectar, and fruit (in situ) also ferment naturally, thereby exposing both terrestrial and arboreal animals to ethanol (see Wiens et al. for two species of treeshrew that consume significant levels of ethanol from the fermented nectar of bertam palms) (55). Overripe fruit that has fallen to the ground is generally older (and more damaged) than ripe fruit picked directly from trees and, as such, has had more time to ferment, potentially leading to higher concentrations of ethanol (ref. 25, and reviewed in ref. 56). Therefore, the transition to an increasingly terrestrial life would likely have exposed the HCG ancestor to fruit with higher ethanol content. In this context, the increased activity of ethanol-metabolizing enzymes (e.g., ADH4) could provide a selective advantage, particularly during a time of large-scale ecological transitions and extinctions brought about by climate change.

Not all terrestrial organisms are expected to consume ethanolic food. For example, some cercopithecoids independently adapted to a terrestrial life after the hominoid–cercopithecoid split (traits that persist in extant lineages such as *Papio* and *Erythrocebus*), but, unlike the hominoid lineages that remained highly frugivorous, cercopithecoids developed a generalist diet including leaves with high amounts of antifeedants (such as the monoterpene geraniol). The cercopithecoid transition to a generalist, herbaceous diet provided these monkeys a less geographically patchy and seasonally fluctuating diet.

There is a critical distinction between the adaptive strategies adopted by hominoids and cercopithecoids, best exemplified by studies of fallback foods among chimpanzees and three extant cercopithecines of the Kibale Forest in Uganda (57). When fruit was available, chimpanzee diet was confined almost exclusively to ripe fruit; when ripe fruit was not available, chimpanzees relied on alternative terrestrial food sources, such as piths and stems that have low antifeedant levels. The three cercopithecines, however, maintained a diverse diet, including leaves even when ripe fruit was abundant. As a consequence, cercopithecines consumed much higher levels of antifeedants (e.g., geraniol). When ripe fruit was not available, the diet of these cercopithecines consisted primarily of unripe fruits, seeds, and leaves (and thus still contained high antifeedant levels). It is possible that competition between terrestrial middle Miocene monkeys and apes may have been the precursor for the digestive differences between these two groups that persist today, with monkeys

adapted to exploit unripe fruit and African apes adapted to exploit over-ripe ones.

Although ripe fruit is generally the preferred food of all hominoids (58–61), those without an ethanol-oxidizing ADH4 (orangutans and gibbons) differ from chimpanzees and gorillas in several important regards. Unlike chimpanzees and gorillas, gibbons and orangutans rely primarily on arboreal fallback foods, and, whereas gibbons and orangutans have unique strategies to buffer against ripe-fruit scarcity, these strategies bear important similarities to the cercopithecines. During times of food scarcity, gibbons increase consumption of unripe fruit, as well as figs, leaves, and flowers (61, 62). Orangutans make greater use of lower quality food regardless of food availability (63), relying heavily and equally on both ripe and unripe fruit even during periods of high food availability (61), and then increasing dietary diversity during food shortages (primarily by increasing consumption of figs, bark, pith, and leaves) (61, 63, 64). Orangutans also store large amounts of energy as fat and therefore can take advantage of periods of fruit superabundance to overcome energy deficits during periods of fruit scarcity (63, 65). The gibbons and orangutans extensive utilization of unripe fruits (the former primarily during times of food shortage) exposes them to higher levels of terpenoid antifeedants relative to ripe and overripe fruit (66). The need to metabolize large terpenoid antifeedants may preclude ADH4 from simultaneously adapting to small substrates like ethanol. If orangutans or gibbons were to exploit fermented fruit, it might require the recruitment of an enzyme not already involved in terpenoid metabolism.

Having adapted to a more terrestrial lifestyle than its predecessors, it is possible that the ancestor of HCG had less access to the fallback food strategies used by arboreal gibbons or orangutans, likely leading to greater selective pressures to exploit alternative fallback food strategies. Our study indicates that the HCG ancestor possessed the capacity for a novel strategy: an ethanol-active digestive ADH4 enzyme that would permit exploitation of food containing increased concentrations of ethanol. Furthermore, because frugivorous vertebrates generally avoid “rotting” (and presumably more ethanolic) fruit when other options are available (reviewed in ref. 6), there may have been little competition for this resource.

If the A294V mutation endowing ADH4 with enhanced ethanol-oxidizing abilities was adaptive in the HCG ancestor, the exploitation of ethanolic food resources might still persist in modern gorillas or chimpanzees under certain conditions, such as food scarcity. Despite numerous anecdotal accounts of primates consuming ethanol-laden food, we are aware of no published accounts that describe such events in detail (much less quantify the amount of ethanol consumed or discern whether the consumption was inadvertent or intentional). However, a report by Ohashi (67) briefly documents wild chimpanzees in Bossou, Guinea consuming significant amounts of ethanol (repeatedly and apparently intentionally) from fermenting palm wine collected from reservoirs placed in the trees by humans. These findings suggest that ethanol consumption, albeit rare, may nonetheless be a significant part of the natural history of modern chimpanzees and support our conclusion that ethanol consumption was important to the common ancestor of humans, chimpanzees, and gorillas.

**Primate Adaptation to Ethanol Beyond Hominoid ADH4.** Thirteen extant and ancestral ADH4 enzymes outside the Hominae lineage were also examined and only one—from the aye aye (an arboreal lemur)—had increased activity toward ethanol (Table S2). The absence of an ethanol-active ADH4 enzyme in cercopithecoids is expected, given their generalist diet (including fallback foods) containing high levels of antifeedants (from grasses, leaves, and/or unripe fruit) (57) rather than overripe and possibly fermented fruit collected from the ground. Further, some of the most terrestrial cercopithecoids, *Erythrocebus*, *Theropithecus*, and *Papio*, live in semiarid savannahs and grasslands where ethanol-containing fruit is rare.

Although ADH4 is involved in the first-pass metabolism of ethanol, other enzymes also contribute to ethanol metabolism (e.g., ADH1 and ADH2, homologs of ADH4 that are abundantly expressed in the liver, and the MEOS). It is therefore possible that species without an ethanol-active ADH4 are adapted to consume significant amounts of ethanol, as seems to be the case for the common treeshrew (55); in such cases, we expect to see adaptations in other metabolic pathways that mitigate the effects of ethanol consumption.

Remarkably, the only primate in our sample with an ethanol-active ADH4 similar to the HCG ancestor, the aye aye, shares the A294V transition that accounts for the increased activity in the HCG ancestor [aye ayes also share a second homoplasy (V117I) with the lineage leading to the HCG ancestor; see *SI Text* for a discussion of its possible significance]. The branch leading to the aye aye is poorly articulated in the phylogeny used to reconstruct ancestral ADH4 sequences (Fig. 1), and more ADH4 sequences must be added to this model to determine when in the past ~45 million y the A294V mutation occurred along the lineage leading to the modern aye aye. Given this uncertainty about the timing of the A294V mutation in the aye aye lineage and limited information about the diet and life history of aye ayes, it is difficult to speculate whether the independently evolved ethanol-active ADH4 in aye aye is also an adaptation to dietary ethanol. However, aye ayes are known to consume arboreal fruit, sap, and nectar (68) (all of which ferment naturally), so it is reasonable to surmise that aye ayes may have consumed dietary ethanol at some point in their natural history. If so, ethanol consumption may persist in extant aye ayes.

**Human ADH4 Variation.** The Ensembl database contained nine human ADH4 missense polymorphisms at the beginning of this study (ca. 2008), but the number of ADH4 polymorphisms reported has since grown to over 50, including several relatively frequent variants. Given the likelihood that human ADH4 is adapted to ethanol, and the observation that one of the three polymorphisms we tested possessed significantly altered activity toward ethanol (*Results*), it is possible that some of the many uncharacterized ADH4 polymorphisms may play an important role in the variability of human ethanol metabolism and risk for developing alcoholism or ethanol-related cancers of the upper gastrointestinal tract.

## Summary

Ancestral reconstructions of ADH4 demonstrate that the ancestor of humans, chimpanzees, and gorillas possessed a novel enzyme with dramatically increased activity toward ethanol, and we suspect that this novel metabolic capacity was adaptive to this hominin ancestor. This transition implies that the genomes of modern human, chimpanzee, and gorilla began adapting at least 10 million y ago to dietary ethanol present in fermenting fruit—a source of ethanol that is remarkably similar in concentration and form (i.e., with food) to the moderate ethanol consumption now recognized to be healthy for many humans (69). This conclusion contrasts with the relatively short amount of time (~9,000 y) (5) since fermentative technology enabled humans to consume beverages (devoid of food bulk) with higher ethanol content than fruit fermenting in the wild. Vestiges of this history may persist in the extant descendants of the HCG ancestor. This history has implications, not only for understanding the forces that shaped hominin terrestrial adaptations, but also for

understanding the medical complexities of human interactions with ethanol today.

## Methods

The evolutionary history of the ADH4 family was reconstructed using ADH4 genes from 28 different mammals, including 17 primates, collected from public databases [National Center for Biotechnology Information (NCBI) and Ensembl] or were generated de novo by RT-PCR of mRNA or genomic DNA extracted from well-preserved tissue samples (*Table S3*). The genomic sequence data for *Tupaia belangeri* in the Ensembl database were lacking exon 6; this gap was filled using sequence data from a closely related species, *Tupaia glis*. The last exon, encoding just eight amino acids, was missing from a few primates (*Table S4*). Because this exon is invariant among primates, the reference sequence was used when the databases did not include data for this exon.

In the acquisition of tissues, ethical standards for the treatment of animals were ensured by the institutions providing samples according to each institution's ethical review board. In general, tissues were collected at necropsy after natural death or were collected initially for research other than that described here.

Genetic material was extracted from these tissues using TRI reagent with minor modifications to the manufacturer's protocol (Sigma). Recovered mRNA was reverse-transcribed, gel-purified, cloned into the TOPO-TA cloning vector (Invitrogen), and then sequenced using Big Dye technology using the service provided by BioBasic.

All protein sequences used in this study are displayed in FASTA format in *Table S4*. These sequences were placed on a tree that represents the standard phylogeny of primate species (Fig. 1). The reference phylogeny used in this study was compiled from various published phylogenies. For nonprimate outgroups, the reference phylogeny was based on the phylogeny of Prasad et al. (70). The phylogeny of Prasad et al. did not include treeshrew, so our reference phylogeny placed treeshrew sister to primates (relative to rodents) according to the phylogeny of Liu et al. (71). The placement of lemurs in our reference phylogeny was based on the phylogeny of Horvath et al. (72). Our reference phylogeny for nonlemur primates was based on the phylogeny of Purvis (73).

Ancestral ADH4 protein sequences were inferred from the extant species sequence data and phylogeny using a maximum-likelihood analysis implemented within the PAML software package (20) by applying a codon model and a discrete gamma distribution for rate variation among sites. When the posterior probability that a particular amino acid occupied a particular site was ≥94%, we assigned that amino acid at that site in the ancestral ADH4. When the posterior probability was <94% at a particular position (as was the case for amino acid 171 at node 44), both forms of the protein were synthesized and examined to determine whether the interpretation of the kinetic results was robust with respect to the ambiguity.

Synthetic genes encoding various ancestral and extant ADH4 proteins were cloned into the pET21 vector (BioBasic) and expressed in the *Escherichia coli* TUNER cell line. The heterologously expressed proteins were isolated following the procedures of Niederhut et al. (74). The activity and kinetic parameters were characterized by measuring the formation of NADH using various alcohols as substrates as in Yin et al. (75), with the exception that BSA was included in the assays at 0.2 mg/mL. The kinetic values we obtained for human ADH4 were similar to those reported in the literature by others (76). Statistical significance was determined using a modification of a two-tailed Student *t* test that does not assume equal variances between the two populations being tested (also known as Welch's *t* test).

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